

PATENT
Attorney Docket 2676-4554.1US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EV 326918765 US
Date of Deposit with USPS: October 8, 2003
Person making Deposit: Christopher Haughton

APPLICATION FOR LETTERS PATENT

for

NOVEL INHIBITORS OF NF-κB ACTIVATION

Inventors:
Rudi Beyaert
Karen Heyninck
Walter Fiers

Attorney:
Scott Dorland, Ph.D.
Registration No. 51,622
TRASKBRITT, P.C.
P.O. Box 2550
Salt Lake City, Utah 84110
(801) 532-1922

TITLE OF THE INVENTION

NOVEL INHIBITORS OF NF-κB ACTIVATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of co-pending application Serial No. 09/702,953, filed October 31, 2000, now U.S. Patent _____, which is a continuation of International Application No. PCT/BE99/00055, filed May 5, 1999, designating the United States of America, published in English as WO 99/57133, the contents of both of which are incorporated by this reference.

REFERENCE TO SEQUENCE LISTING

[0002] This application contains a Sequence Listing appendix and a computer readable form, both of which are hereby incorporated by reference.

TECHNICAL FIELD

[0003] This invention relates generally to biotechnology, and more particularly to novel inhibitors of the Nuclear factor kappa B (NF-κB) activating pathway useful in the treatment of NF-κB related diseases and/or in the improvement of anti-tumor treatments. The invention also relates to nucleic acids coding for the novel inhibitors. The invention relates further to the use of polypeptides, derived from these inhibitors in the treatment of NF-κB related diseases and/or cancer. Furthermore, the invention concerns pharmaceutical preparations, comprising the novel inhibitors or the polypeptides, derived from these inhibitors and methods of screening with these compounds.

BACKGROUND

[0004] NF-κB is an ubiquitously expressed transcription factor that controls the expression of a diverse range of genes involved in inflammation, immune response, lymphoid differentiation, growth control and development. NF-κB resides in the cytoplasm as an inactive dimer consisting of p50 and p65 subunits, bound to an inhibitory protein known as IκB. The latter becomes phosphorylated and degraded in response to various environmental stimuli, such as pro-inflammatory

cytokines, viruses, lipopolysaccharides, oxidants, UV light and ionizing radiation. This allows NF- κ B to translocate to the nucleus where it activates genes that play a key role in the regulation of inflammatory and immune responses, including genes that encode pro-inflammatory cytokines (IL-1 β , TNF, GM-CSF, IL-2, IL-6, IL-11, IL-17), chemokines (IL-8, RANTES, MIP-1 α , MCP-2), enzymes that generate mediators of inflammation (NO synthetase, cyclo-oxygenase), immune receptors (IL-2 receptor) and adhesion molecules (ICAM-1, VCAM-1, E-selectin). Some of these induced proteins can in turn activate NF- κ B, leading to the further amplification and perpetuation of the inflammatory response. Recently, NF- κ B has been shown to have an anti-apoptotic role in certain cell types, most likely by inducing the expression of anti-apoptotic genes. This function may protect tumor cells against anti-cancer treatments and opens the possibility to use NF- κ B inhibiting compounds to sensitize the tumor cells and to improve the efficiency of the anti-cancer treatment.

[0005] Because of its direct role in regulating responses to inflammatory cytokines and endotoxin, activation of NF- κ B plays an important role in the development of different diseases such as (Barnes and Karin, 1997): chronic inflammatory diseases, *i.e.*, rheumatoid arthritis, asthma and inflammatory bowel disease (Brand et al., 1996); acute diseases, *i.e.*, septic shock (Remick, 1995); Alzheimer's disease where the β -amyloid protein activates NF- κ B (Behl et al., 1997); atherosclerosis, where NF- κ B may be activated by oxidized lipids (Brand et al., 1997); autoimmune diseases, *i.e.*, such as systemic lupus erythematosus (Kalschmidt et al., 1994); or cancer by up-regulating certain oncogenes or by preventing apoptosis (Luque et al., 1997). In addition, NF- κ B is also involved in viral infection since it is activated by different viral proteins, such as occurs upon infection with rhinovirus, influenza virus, Epstein-Barr virus, HTLV, cytomegalovirus or adenovirus. Furthermore, several viruses such as HIV have NF- κ B binding sites in their promoter/enhancer regions (Mosialos, 1997).

[0006] Because of the potential role of NF- κ B in many of the above mentioned diseases, NF- κ B and its regulators have drawn much interest as targets for the treatment of NF- κ B related diseases. Glucocorticoids are effective inhibitors of NF- κ B, but they have endocrine and metabolic side effects when given systematically (Barnes et al., 1993). Antioxidants may represent another class of NF- κ B inhibitors, but currently available antioxidants, such as acetyl-cysteine are relatively weak and unspecific (Schreck et al., 1991). Aspirin and sodium salicylate also inhibit activation of NF- κ B, but only at relatively high concentrations (Kopp and Gosh, 1994). There are some natural

inhibitors of NF-κB such as glyotoxin, derived from Aspergillus, but these compounds are too toxic to be used as a drug (Pahl et al., 1996). Finally, there may be endogenous inhibitors of NF-κB, such as IL-10, that blocks NF-κB through an effect on IκB (Wang et al., 1995). However, total inhibition of NF-κB in all cell types for prolonged periods is unwanted, because NF-κB plays a crucial role in the immune response and other defensive responses.

[0007] An important role in the induction of NF-κB by TNF and IL1 has recently been demonstrated for TNF-receptor associated factors, TRAF2 and TRAF6, which are recruited to the stimulated TNF-receptor and IL-1 receptor, respectively (Rothe et al., 1995; Cao et al., 1996). Over expression of TRAF2 or TRAF6 activates NF-κB, whereas dominant negative mutants inhibit TNF or IL-1 induced activation of NF-κB in most cell types. TRAF2 knock out studies have recently shown that TRAF2 is not absolutely required for NF-κB activation, presumably because of redundancy within the TRAF family (Yeh et al. 1997). The TRAF induced signaling pathway to NF-κB was further resolved by the identification of the TRAF-interacting protein NIK, which mediates NF-κB activation upon TNF and IL-1 stimulation by association and activation of IκB kinase-α and -β (IKK) (Malinin et al, 1997; Regnier et al., 1997; DiDonato et al., 1997; Zandi et al., 1997; Woronicz et al., 1997). The latter are part of a large multi-protein NF-κB activation complex and are responsible for phosphorylation of IκB, leading to its subsequent degradation and to translocation of released, active NF-κB to the nucleus. This allows a more specific inhibition of NF-κB activation by stimuli (including TNF and IL-1) that activate TRAF pathways. Based on this principle, WO 97/37016 discloses the use of NIK and other TRAF interacting proteins for the modulation of NF-κB activity.

[0008] Another protein that can associate with TRAF2 is the zinc finger protein A20 (Song et al., 1996). The latter is encoded by an immediate early response gene induced in different cell lines upon stimulation by TNF or IL-1 (Dixit et al, 1990). Interestingly, over expression of A20 blocks both TNF and IL-1 induced NF-κB activation (Jaattela et al., 1996). However, the mechanism by which A20 blocks NF-κB activation is totally unknown. In contrast to NIK, A20 does not seem to act directly on IκB resulting in alternative pathway to modulate NF-κB activation.

[0009] De Valck et al. (1997) isolated an A20 binding protein, so-called 14-3-3, using a yeast two-hybrid assay and demonstrated that NF-κB inhibition was independent from the binding of A20 to 14-3-3.

SUMMARY OF THE INVENTION

[0010] It is shown herein that other new A20 interacting proteins unexpectedly can modulate and/or inhibit NF-κB activation.

[0011] The invention includes an isolated functional protein comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO:2, or comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO:19, or, in the alternative, comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO:6.

[0012] More specifically, the functional protein comprises an amino acid sequence with 70-100% homology to the amino acids 54-647 of SEQ ID NO:2, even more specifically the functional protein comprises an amino acid sequence with 70-100% homology to the amino acids 390-647 of SEQ ID NO:2, and/or comprising an amino acid sequence with 70-100% homology to the amino acids 420-647 of SEQ ID NO:2.

[0013] Homology, in this context, means identical or similar to the referenced sequence, while known replacements/modifications of any of the amino acids provided, are included as well. A homology search in this respect can be performed with the BLAST-P (Basic Local Alignment Search Tool) program well known to a person skilled in the art. For the corresponding nucleic acid sequence homology is referred to the BLASTX and BLASTN programs known in the art.

[0014] One aspect of the invention is to offer novel modulators and/or inhibitors of TNF and/or IL-1 induced NF-κB activation pathways.

[0015] An important embodiment of the invention is a protein comprising at least the amino acids of SEQ ID NO:2.

[0016] Another embodiment of the invention is a protein comprising at least the amino acids 54-647 of SEQ ID NO:2, as represented in SEQ ID NO:19.

[0017] A further embodiment of the invention is a protein comprising at least the amino acids of SEQ ID NO:6.

[0018] A further aspect of the invention is the use of a protein comprising the amino acids 420-647 of SEQ ID NO:2 to modulate and/or inhibit the NF-κB related pathway, especially the TNF and/or IL-1 induced pathways.

[0019] In addition, the invention concerns the use of a protein, comprising the consensus sequence shown in SEQ ID NO:8 and/or SEQ ID NO:9, to modulate and/or inhibit the TNF and/or IL-1 induced, NF- κ B related pathway.

[0020] Another aspect of the invention is the use of these proteins in a screening method to screen compounds that interfere with the interaction of these protein(s) with other protein components of the NF- κ B related pathway.

[0021] Another embodiment of the invention is the use of the above mentioned proteins, or the use of protein components screened by the above mentioned method to sensitize tumor cells and/or improve the anti-cancer treatment.

[0022] The present invention also relates to a method for identifying and obtaining an activator or inhibitor of A20 interacting protein(s) comprising the steps of:

- (a) combining a compound to be screened with a reaction mixture containing the protein of the invention and a read out system capable of interacting with the protein under suitable conditions;
- (b) maintaining the reaction mixture in the presence of the compound or a sample comprising a plurality of compounds under conditions which permit interaction of the protein with the read out system;
- (c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation of the read out system.

[0023] The term "read out system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant DNA molecules and marker genes as described above.

[0024] The term "plurality of compounds" in a method of the invention is understood as a plurality of substances which may be identical.

[0025] The compound or plurality of compounds may be comprised in, for example, samples, *e.g.*, cell extracts from animals or microorganisms. Furthermore, the compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating A20 interacting proteins. The reaction mixture may be a cell free extract or may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person skilled in the art and are, for

example, generally described in Alberts et al., Molecular Biology of the Cell, (3rd ed. 1994). The plurality of compounds may be, for instance, added to the reaction mixture or culture medium, or may be injected into the cell.

[0026] If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating A20 interacting proteins. Additionally, one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample. The method can then be repeated with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of, or only one substance(s). Preferably, the sample comprises substances of similar chemical and/or physical properties, and most preferably the substances are identical. The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, *Nature Medicine* 1 (1995), 879-880; Hupp, *Cell* 83 (1995), 237-245; Gibbs, *Cell* 79 (1994), 193-198 and references cited *supra*).

[0027] The invention also relates to a DNA sequence encoding the referenced proteins or a DNA sequence encoding an immunologically active and/or functional fragment of such a protein, selected from the group consisting of:

- (a) DNA sequences comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO:2;
- (b) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO:1;
- (c) DNA sequences hybridizing with the complementary strand of a DNA sequence as defined in (a) or (b) and encoding an amino acid sequence which is at least 70% identical to the amino acid sequence encoded by the DNA sequence of (a) or (b);
- (d) DNA sequences, the nucleotide sequence of which is degenerated as a result of the genetic code to a nucleotide sequence of a DNA sequence as defined in any one of (a) to (c); and

- (e) DNA sequences encoding a fragment of a protein encoded by a DNA sequence of any one of (a) to (d).

[0028] Thus, the invention consists of DNA molecules, also called nucleic acid sequences, encoded for the above mentioned proteins preferably a nucleic acid sequence, with 70-100% homology to the DNA sequence depicted in SEQ ID NO:1, and/or a nucleic acid sequence with 70-100% homology to the DNA sequence depicted in SEQ ID NO:5.

[0029] Homology in this context means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of the nucleic acid molecules are, for example, variations of the nucleic acid molecules which represent modifications having the same biological function. In particular, the modifications encode proteins with the same or substantially the same biological function. They may be naturally occurring variations, such as sequences from other varieties or species, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variations may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants.

[0030] The proteins encoded by the various derivatives and variants of the above-described nucleic acid molecules have similar common characteristics, such as biological activity, molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature optimum, stability, solubility, spectroscopic properties, etc.

[0031] The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory (1989) N.Y.

[0032] Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

[0033] In a preferred embodiment, the nucleic acid molecule present in the vector is operably linked to (a) control sequence(s) which allow the expression of the nucleic acid molecule in prokaryotic and/or eukaryotic cells.

[0034] The term "control sequence" refers to regulatory DNA sequences which are necessary to affect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes, control sequences generally include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components that are necessary for expression, and may also include additional advantageous components.

[0035] The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is known to a skilled person that double-stranded nucleic acid is used.

[0036] Thus, the vector of the invention is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can, for instance, be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art.

[0037] The present invention furthermore relates to host cells comprising a vector as described herein or a nucleic acid molecule according to the invention wherein the nucleic acid molecule is foreign to the host cell.

[0038] By "foreign" is meant that the nucleic acid molecule is either heterologous or homologous with respect to the host cell. "Heterologous" means derived from a cell or organism with a different genomic background. "Homologous" means located in a different genomic environment than the naturally occurring counterpart of the nucleic acid molecule. Thus, if the

nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of the host cell, but it is surrounded by different genes. In this case, the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule, according to the invention, which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extra-chromosomally. It is also possible that the nucleic acid molecule of the invention can be used to restore or create a mutant gene via homologous recombination (Paszkowski (ed.), *Homologous Recombination and Gene Silencing in Plants*, (Kluwer Academic Publishers 1994)).

[0039] The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*.

[0040] The invention also includes a method for preparing A20 interacting proteins which method comprises the cultivation of host cells that due to the presence of a vector or a nucleic acid molecule according to the invention, are able to express such a protein under conditions which allow expression of the protein and thus recovery of the so-produced protein from the culture.

[0041] The term "expression" means the production of a protein or nucleotide sequence in the cell. However, the term also includes expression of the protein in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications. Depending on the specific constructs and conditions used, the protein may be recovered from the cells, from the culture medium or from both. For the person skilled in the art, it is well known that it is not only possible to express a native protein, but also to express the protein as fusion polypeptides or to add signal sequences directing the protein to specific compartments of the host cell, for example, ensuring secretion of the peptide into the culture medium, etc. Furthermore, such a protein and fragments thereof can be chemically synthesized and/or modified according to standard methods.

[0042] The terms "protein" and "polypeptide" used in this application are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition

of polypeptide. This term also refers to and includes post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

[0043] The present invention furthermore relates to proteins encoded by the nucleic acid molecules according to the invention or produced or obtained by the methods described herein, and to functional and/or immunologically active fragments of such A20 interacting proteins. The proteins and polypeptides of the present invention are not necessarily translated from a designated nucleic acid sequence. The polypeptides may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from a suitable viral system. The polypeptides may include one or more analogs of amino acids, phosphorylated amino acids or unnatural amino acids. Methods of inserting analogs of amino acids into a sequence are known in the art. The polypeptides may also include one or more labels, which are known to those skilled in the art. In this context, it is also understood that the proteins according to the invention may be further modified by conventional methods known in the art. By providing the proteins according to the present invention it is also possible to determine fragments which retain biological activity, namely the mature, processed form. This allows the construction of chimeric proteins and peptides comprising an amino sequence derived from the protein of the invention, which is crucial for its binding activity. The other functional amino acid sequences may be either physically linked by, for example, chemical means to the proteins of the invention or may be fused by recombinant DNA techniques well known in the art.

[0044] The term "functional fragment of a sequence" or "functional part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a

maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

[0045] Furthermore, folding simulations and computer redesign of structural motifs of the protein of the invention can be performed using appropriate computer programs (Olszewski, *Proteins* 25 (1996), 286-299; Hoffman, *Comput. Appl. Biosci.* 11 (1995), 675-679). Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed peptide and protein models (Monge, *J. Mol. Biol.* 247 (1995), 995-1012; Renouf, *Adv. Exp. Med. Biol.* 376 (1995), 37-45). In particular, the appropriate programs can be used for the identification of interactive sites of the inventive protein , its receptor, its ligand or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, *Immunomethods* 5 (1994), 114-120. Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, *Biochem. Soc. Trans.* 22 (1994), 1033-1036; Wodak, *Ann. N. Y. Acad. Sci.* 501 (1987), 1-13; Pabo, *Biochemistry* 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used for, e.g., the preparation of peptidomimetics of the protein of the invention or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein (Benkirane, *J. Biol. Chem.* 271 (1996), 33218-33224). For example, incorporation of easily available achiral amino acid residues into a protein of the invention or a fragment thereof results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptidomimetic (Banerjee, *Biopolymers* 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, *Biochem. Biophys. Res. Commun.* 224 (1996), 327-331). Appropriate peptidomimetics of the protein of the present invention can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, e.g., for their binding and immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art. See, e.g., Ostresh, *Methods in Enzymology* 267 (1996), 220-234; Dorner, *Bioorg. Med. Chem.* 4 (1996), 709-715.

[0046] Furthermore, a three-dimensional and/or crystallographic structure of the protein of the invention can be used for the design of peptidomimetic inhibitors of the biological activity of the

protein of the invention (Rose, *Biochemistry* 35 (1996), 12933-12944; Rutenber, *Bioorg. Med. Chem.* 4 (1996), 1545-1558).

[0047] Furthermore, the present invention relates to antibodies specifically recognizing a A20 interacting protein according to the invention or parts, *i.e.* specific fragments or epitopes, of such a protein. The antibodies of the invention can be used to identify and isolate other A20 interacting proteins and genes in any organism. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Kohler and Milstein, *Nature* 256 (1975), 495, and Galfre, *Meth. Enzymol.* 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, for example, in Harlow and Lane "*Antibodies, A Laboratory Manual*", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of proteins according to the invention. Additionally, the antibodies can be used for the monitoring of the synthesis of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the protein according to the invention. For example, surface plasmon resonance as employed in the BIACore system can be used to increase the efficiency of phage antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of the protein of the invention (Schier, *Human Antibodies Hybridomas* 7 (1996), 97-105; Malmborg, *J. Immunol. Methods* 183 (1995), 7-13). In many cases, the binding phenomena of antibodies to antigens is equivalent to other ligand/anti-ligand binding.

[0048] The invention also relates to a diagnostic composition comprising at least one of the nucleic acid molecules, vectors, proteins, antibodies or compounds described herein and, optionally, a suitable means for detection.

[0049] The diagnostic compositions may be used in methods for detecting expression of related A20 interacting proteins. This is accomplished by detecting the presence of the corresponding mRNA which comprises isolation of mRNA from a cell, contacting the mRNA obtained with a probe comprising a nucleic acid probe as described herein under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and/or detecting the expression

of the protein in the cell. Further methods of detecting the presence of a protein according to the present invention comprises immunotechniques well known in the art, for example, enzyme-linked immunosorbent assays.

[0050] The invention also relates to a pharmaceutical composition comprising one or more compounds, obtained by a screening method described herein, in a biologically active amount, for the treatment of NF- κ B related diseases such as respiratory disorders, particularly adult respiratory distress syndrome, allograft rejection, chronic inflammatory diseases such as rheumatoid arthritis, asthma or inflammatory bowel disease, and/or autoimmune diseases such as systemic lupus erythematosis.

[0051] In another aspect, the invention relates to a pharmaceutical composition comprising one or more of the A20 interacting proteins in a biologically active amount, for the treatment of NF- κ B related diseases such as respiratory disorders, particularly adult respiratory distress syndrome, allograft rejection, chronic inflammatory diseases such as rheumatoid arthritis, asthma or inflammatory bowel disease, and/or autoimmune diseases such as systemic lupus erythematosis.

[0052] The invention also concerns a pharmaceutical composition comprising one or more of the A20 interacting proteins and/or one or more of the above mentioned compounds in a biologically active amount, for a treatment to sensitize tumor cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0053] FIG. 1: Tissue distribution of ABIN transcripts. A Northern blot of poly(A)+ RNA (2 μ g per lane) of various murine tissues (Clontech) was probed with the fragment of ABIN cloned by two hybrid analysis covering the C-terminal sequences ABIN (390-599). RNA size markers are indicated in kB. Expression of β -actin served as a control for the quantity of RNA loaded.

[0054] FIG. 2: Co-immunoprecipitation of A20 and ABIN after transient transfection of the encoding plasmids of E-tagged ABIN and Green Fluorescent Protein (GFP), GFP-A20, GFP-A20(369-775), GFP-A20(1-368) or an empty expression vector as a negative control in 239T cells. Immunoprecipitation (upper panel) was performed with anti-GFP antibody and Western blot detection with anti E-tag antibody. To control expression levels of ABIN, 10 μ l aliquots of lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot detection with anti-E-tag antibody (lower panel).

[0055] FIG. 3: Co-immunoprecipitation of the C-terminal fragment of ABIN lacking the putative leucine zipper structure with GFP-A20 upon transient over expression in 293T cells. Immunoprecipitation and expression levels were detected as described for full length ABIN and are shown in the upper and lower panel, respectively.

[0056] FIG. 4 is a bar graph depicting the effect of ABIN or fragments of ABIN on the TNF- and IL-1-induced activation of NF- κ B, as measured by reporter gene activity. 293T cells were transiently transfected with 100 ng pUT651, 100 ng pNFconluc and 100 ng expression plasmid and stimulated with hTNF (1000 IU/ml) or mIL1 β (20 ng/ml) during 6 hours. As a control, 100 ng of plasmids encoding GFP or GFP-A20 were transfected.

[0057] FIG. 5 is a bar graph depicting the effect of transient transfection of sub-optimal quantities of expression plasmids encoding A20 (5ng) and ABIN (20 ng) on TNF mediated NF- κ B induction in 293T cells. In both experiments, standard deviations were less than 10 %.

[0058] FIG. 6 is a bar graph depicting the effect of ABIN or fragments of ABIN on the TPA-induced activation of NF- κ B, as measured by reporter gene activity. 293T cells were transiently transfected with 100 ng pUT651, 100 ng pNFconluc and 100 ng expression plasmid and stimulated with TPA (200 ng/ml) during 6 hours. As a control, 100 ng of plasmids encoding GFP or GFP-A20 were transfected.

[0059] FIG. 7 is a bar graph depicting the effect of ABIN2 on the TNF- and TPA-induced activation of NF- κ B, as measured by reporter gene activity. 293T cells were transiently transfected with 100 ng pUT651, 100 ng pNFconluc and 600 ng expression plasmid and stimulated with hTNF (1000IU/ml) or TPA (200 ng/ml) during 6 hours. As a control, 600 ng of plasmid encoding GFP or GFP-A20 was transfected.

[0060] FIG. 8 is a bar graph depicting the effect of full length ABIN on NF- κ B activation in 293T cells induced by over expression of TRADD, RIP, TRAF2, NIK or p65 after transfection of 300 ng of their encoding plasmids, together with 100 ng pUT651, 100 ng pNFconluc and 500 ng pCAGGS-ABIN. Cells were lysed 24 hours after transfection, and luciferase and β -galactosidase activity were measured.

[0061] FIG. 9 specifically is a bar graph depicting the effect of truncated ABIN containing the leucine zipper structure (ABIN(390-647)) on TRADD, RIP, TRAF2 or NIK induced NF- κ B activation. In both experiments, standard deviations were less than 10 %.

[0062] FIGs. 10, 11 and 12: As an overview, these figures illustrate the effect of site specific mutations in two regions of ABIN on its binding with A20 and on its inhibition of NF- κ B activation.

[0063] FIG. 10 shows the co-immunoprecipitation of mutated ABIN with A20 after transient expression of these genes in 293T cells. Cells were transfected with the plasmids pCAGGS-GFP or pCAGGS-GFP/A20 together with the plasmids encoding ABIN or its site specific mutants (ABIN-MUT1, ABIN-MUT2, ABIN-MUT3 or ABIN-MUT4). Lysates of these cells were immunoprecipitated with polyclonal anti-GFP antibody and separated on SDS-PAGE. Western blot analysis was performed with monoclonal anti-E-tag antibody to look for the Co-immunoprecipitation of ABIN or its mutants (upper panel). Lower panels show total expression levels of GFP, GFP/A20 and ABIN. In this case, a fraction of the total lysate was separated by SDS-PAGE and expression was detected with anti-GFP or anti-E-tag antibodies.

[0064] FIG. 11 is a bar graph depicting the effect of mutated ABIN on TNF-induced NF- κ B activation. 293T cells were transiently transfected with 100 ng pUT651, 100 ng pNFconluc and 200 ng expression plasmid as indicated and stimulated with TNF (1000 IU/ml) during 6 hours. Cell extracts were analyzed for luciferase and β -galactosidase activity and plotted as luc/gal, which is representative for NF- κ B activity. Each value is the mean (N=3) with standard deviations less than 10%.

[0065] FIG. 12 is a bar graph depicting the dominant negative effect of ABIN-MUT2, ABIN-MUT3 and ABIN-MUT4 on the NF- κ B inhibiting function of ABIN. 293T cells were transiently transfected with 100 ng pUT651, 100 ng pNFconluc and 200 ng pCAGGS-ABIN or empty vector. In addition, 600 ng of the expression vectors encoding ABIN-MUT2, ABIN-MUT3, ABIN-MUT4 or empty vector were co-transfected, as indicated. Cells were stimulated with TNF (1000 IU/ml) during 6 hours. Cell extracts were analyzed for luciferase and β -galactosidase activity and plotted as luc/gal.

DETAILED DESCRIPTION OF THE INVENTION

[0066] The following definitions are provided in order to further illustrate and define the meaning and scope of the various terms used in the current description.

[0067] The term "treatment", "treating" or "treat" means any treatment of a disease in a mammal, including: (1) preventing the disease causing the clinical symptoms of the disease not to develop; (2) inhibiting the disease arresting the development of the clinical symptoms; and/or (3) relieving the disease causing the regression of clinical symptoms.

[0068] The term "effective amount" means a dosage sufficient to provide treatment for the disease state being treated. This will vary depending on the patient, the disease and the treatment being effected.

[0069] "Capable of interacting" means that a protein can form a complex with another protein, as can be measured using a yeast two hybrid system, or with co-immunoprecipitation, or with equivalent systems known to people skilled in the art.

[0070] "Functional" protein or fragment means a protein or fragment that is capable to interact with the zinc finger protein A20, or with another protein of the NF- κ B related pathway.

[0071] "Protein A20" ("A20") means the TNF induced zinc finger protein, described by (Dixit et al., 1990; Oripipari et al., 1990; Tewari et al., 1995), or an active fragment thereof, such as the zinc finger containing part (amino acids 387-790 of human A20; amino acids 369-775 of murine A20).

[0072] The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms refer only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog. Preferably, the DNA sequence of the invention comprises a coding sequence encoding the above defined A20 interacting protein.

[0073] A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

[0074] "Consensus sequence" means a stretch of at least 15 amino acids, showing 50-100% homology, preferably between 70-100% homology between ABIN and ABIN2.

[0075] "Compound" means any chemical or biological compound, including simple or complex inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates or nucleic acids, that interfere in the binding between a protein depicted in SEQ ID NOS:2, 19, 6, 8 or 9 with a compound of the NF- κ B related pathway, such as A20.

[0076] As used herein, the term "composition" refers to any composition such as a pharmaceutical composition comprising an active ingredient of an isolated functional protein according to the present invention. This may be performed in the presence of suitable excipients known to the skilled man and may be administered in the form of any suitable composition as detailed below, and by any suitable method of administration within the knowledge of a skilled man. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable excipient. Such excipients are inherently nontoxic and non-therapeutic. Examples of such excipients are saline, Ringer's solution, dextrose solution and Hank's solution. Non-aqueous excipients such as fixed oils and ethyl oleate may also be used. A preferred excipient is 5% dextrose in saline. The excipient may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

[0077] The isolated functional protein of the invention is administered at a concentration that is therapeutically effective to prevent allograft rejection, GVHD, allergy and autoimmune diseases. The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that the isolated functional protein is given at a dose between 1 μ g/kg and 10 mg/kg, more preferably between 10 μ g/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous short time infusion (during 30 minutes) may also be used. The compositions comprising the isolated functional protein according to the invention may be infused at a dose between 5 and 20 μ g/kg/minute, more preferably between 7 and 15 μ g/kg/minute.

[0078] The "therapeutically effective amount" of the isolated functional protein needed in a specific case, according to the invention, should be determined as being the amount sufficient to cure

the patient in need of treatment or at least to partially arrest the disease and its complications. Amounts effective for such use will depend on the severity of the disease and the general state of the patient's health. Single or multiple administrations may be required depending on the dosage and frequency as required and tolerated by the patient.

[0079] With regard to the use of the isolated functional protein of the present invention to prevent allograft rejection, it should be stressed that the proteins of the present invention or the compositions comprising the same may be administered before, during or after the organ transplantation as is desired from case to case. In case the protein or the compositions comprising the same are administered directly to the host, treatment will preferably start at the time of the transplantation and continue afterwards in order to prevent the activation and differentiation of host T cells against the MHC on the allograft. In case the donor organ is ex vivo perfused with the functional protein according to the invention or the compositions comprising the same, treatment of the donor organ ex vivo will start before the time of the transplantation of the donor organ in order to prevent the activation and differentiation of host T cells against the MHC on the allograft.

[0080] The invention is hereunder further explained by way of examples without being restrictive in the scope of the current invention.

EXAMPLES

Example 1: Isolation of the novel inhibitors

[0081] The novel inhibitors of the NF- κ B pathway were isolated using a yeast two-hybrid assay, with protein A20 as bait. The yeast two-hybrid assay was purchased from Clontech Laboratories (Palo Alto, CA). The screening of an L929r2 cDNA library with pAS2-A20 was described previously (De Valck et al., 1997). Yeast colonies expressing interacting proteins were selected by growth on minimal media lacking Tryptophan, Leucine and Histidine, in the presence of 5 mM 3-amino-1,2,4-triazole and by screening for β -gal activity. Plasmid DNA was extracted from the positive colonies and the pGAD424 vectors encoding candidate A20 interacting proteins were recovered by electroporation in the *E. coli* strain HB101 and growth on media lacking Leucine.

[0082] From 1.3×10^6 transformants, 11 clones expressed A20 interacting proteins, including A20 itself (De Valck et al., 1996) and 14-3-3 proteins (De Valck et al., 1997). Three clones contained C-terminal fragments of the same cDNA encoding an unknown protein that

herewith is named A20 Binding Inhibitor of NF- κ B activation (ABIN) and 1 clone contained the C-terminal fragment (1136 bp) of an unknown protein that herewith is called ABIN2.

[0083] Full length ABIN cDNA was subsequently isolated from the L929r2 cDNA library by colony hybridization (De Valck et al., 1996) with an ABIN fragment (corresponding to amino acids 390-599) cloned by two-hybrid analysis as a probe. Several cDNA's were isolated and in the longest cDNA stop codons were identified in all three reading frames 5' of a potential initiator methionine. Two different splice variants were found of approximately 2800 and 2600 nucleotides long, with an open reading frame of 1941 and 1781 nucleotides respectively, initiating at two different methionines (ABIN (1-647) (SEQ ID NO:2) and ABIN (54-647) (SEQ ID NO:19)). These cDNA's encode proteins of 72 and 68 kDa containing an amphipathic helix with 4 consecutive repeats of a leucine followed by 6 random amino acids residues characteristic of a leucine zipper structure.

[0084] Full length cDNA of ABIN2 was isolated from murine heart by 5' RACE (SMART PCR cDNA synthesis kit, Clontech), using a 3' primer hybridizing to an EST clone (572231) which corresponds to the ABIN2 fragment isolated by two hybrid analysis, but with 507 extra nucleotides at the 5' end. A 1,967 nucleotide long cDNA was isolated (SEQ ID NO:5), with an open reading frame of 1,290 nucleotides long, encoding a protein of 430 amino acids (SEQ ID NO:6).

Example 2 : Expression pattern of ABIN and ABIN2.

[0085] Northern blot analysis revealed that both ABIN and ABIN2 are expressed in all murine tissues tested (heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis: see FIG. 1; only the data for ABIN are shown). ABIN is present as mRNA of approximately 2800 bp which is in accordance with the length of the cloned full length cDNA. In contrast to A20, ABIN mRNA is constitutively expressed in both TNF-sensitive and TNF-resistant subclones derived from the parental cell line L929s, irrespective of TNF stimulation.

[0086] ABIN2 is present as mRNA of approximately 2,000 bp, which is in accordance with the length of the cloned, full length cDNA.

Example 3: Study of the interaction of the ABIN and ABIN2 proteins and protein fragments with A20.

[0087] Full length ABIN(1-647) and ABIN(54-647) were able to bind A20 in a yeast two hybrid assay, confirming the original interaction found with the 3 C-terminal fragments ABIN(390-599), ABIN(249-647) and ABIN(312-647). The latter contain the putative leucine zipper protein interaction motif (397-420).

[0088] Further analysis was carried out by co-immunoprecipitation. The eukaryotic plasmids for ABIN and its fragments as well as for ABIN2 were made by inserting the corresponding PCR fragment in frame with an N-terminal E-tag into the mammalian expression plasmid pCAGGS (Niwa et al., 1991). cDNA encoding mutant GFP(S65T) and a fusion protein of GFP(S65T) with murine A20 were also cloned in pCAGGS.

[0089] 2×10^6 human embryonic kidney 293T cells were plated on 10-cm Petri dishes and transiently transfected with the suitable plasmids by calcium phosphate-DNA co-precipitation. 24 hours after transfection, cells were lysed in $500\mu\text{l}$ of lysis buffer (50 mM Hepes pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40 and 5 mM EDTA). Lysates were incubated with $5\mu\text{l}$ of rabbit anti-GFP antibody (Clontech) and immunocomplexes were immobilized on protein A-trisacryl (Pierce). The latter was washed twice with lysis buffer and twice with lysis buffer containing 1 M NaCl. Co-precipitating proteins were separated by SDS-PAGE and revealed by Western blotting with mouse anti-E-tag antibody (Pharmacia).

[0090] Full length ABIN as well as the C-terminal fragment lacking the leucine zipper motif (ABIN(420-647)) was still able to co-immunoprecipitate with A20 in 293T cells that were transiently transfected with an expression plasmid for chimeric GFP-A20 protein and full length or truncated ABIN with an N-terminal E-tag (FIG. 2). Interaction of ABIN with A20 required the C-terminal, zinc finger containing part of A20 (A20(369-775)). This domain was shown previously to be required for dimerization of A20 and for the interaction of A20 with 14-3-3 protein (De Valck et al., 1996; De Valck et al., 1997). In contrast, the N-terminal part of human A20 (A20(1-386)) was previously shown to interact with TRAF2 (Song et al., 1996), suggesting that A20 acts as an adapter protein between TRAF2 and ABIN. The interaction between A20 and ABIN was not influenced by stimulation with TNF.

[0091] To characterize the sub-cellular distribution of ABIN, we transiently transfected GFP-A20- and E-tagged ABIN cDNA in 293T cells and analyzed their expression by means of GFP fluorescence and immunofluorescence via the anti-E tag antibody. 4×10^5 293T cells were seeded

on cover slips in 6-well plates and transfected with 1 μ g plasmid DNA. 24 hours after transfection, cells were fixed on the cover slips with 3% paraformaldehyde. Upon permeabilization with 1% Triton X-100, the cells were incubated for 2 hours with mouse anti-E-tag antibody (1/1000) followed by a second incubation with anti-mouse Ig antibody coupled to biotin (Amersham, 1/1000). After subsequent incubation with streptavidin coupled to Texas Red (Amersham), fluorescence can be analyzed via fluorescence microscopy (Zeiss, Axiophot) using a filter set with excitation at 543 nm and emission at 600nm. In the same cells, fluorescence of GFP can be detected at a different wave length, namely absorption at 485 nm and emission at 510 nm.

[0092] ABIN co-localized with A20 throughout the cytoplasm, both in unstimulated and in TNF stimulated cells. This observation makes the existence of regulatory redistribution events rather unlikely.

Example 4: Sequence analysis of the cDNA's.

[0093] Nucleotide sequence analysis was carried out using cycle sequencing on an ABI373A sequencer (Applied Biosystems, Foster City, CA). The sequence of full length ABIN is shown in SEQ ID NO:1; the sequence of ABIN2 is shown in SEQ ID NO:5.

[0094] Database similarity searches (BLAST) showed that ABIN is the murine homologue of the partial human cDNA encoding a protein with an unknown function (Genbank accession number D30755; Nagase et al., 1995). Moreover, ABIN shows homology with a partial human immunodeficiency virus (HIV) Nef interacting protein, NIP40-1 (now called Naf1=nef associated factor 1; Fukushi et al., FEBS Letters, 442 (1999), 83-88). HIV-Nef contributes substantially to disease pathogenesis by augmenting virus replication and markedly perturbing T-cell function. Interestingly, the effect of Nef on host cell activation has been explained in part by its interaction with specific cellular proteins involved in signal transduction (Harris, 1996) of which ABIN might be an example.

[0095] There are no proteins in the database that are clearly homologous with ABIN2. However, by comparing ABIN2 with ABIN, one can define two homologous regions and derive two consensus sequences (SEQ ID NO:8 and SEQ ID NO:9)) that may be important for the interaction of these proteins with A20, and/or for their further function in signal transduction. A consensus sequence of ABIN and ABIN2 is found from amino acids 423 to 441 and 475 to 495 of SEQ ID

NO:2 for ABIN, and from amino acids 256 to 274 and 300 to 320 of SEQ ID NO:6 for ABIN2. The consensus sequence illustrates 22 amino acids from ABIN and ABIN2 that overlap with one another.

Example 5: Role of ABIN, ABIN fragments and/or ABIN2 in the TNF-, IL-1- and/or TPA-induced transduction pathway leading to NF- κ B activation, as measured by reporter gene activity.

[0096] The construction of the ABIN, ABIN-fragments and ABIN2 plasmids was carried out as described above. The plasmid pNFconluc, encoding a luciferase reporter gene driven by a minimal NF- κ B responsive promoter described by Kimura et al. (1986) and the plasmid pUT651, encoding β -galactosidase was obtained from Eurogentec (Seraing, Belgium). NF- κ B activity was determined by the NF- κ B dependent expression of a luciferase reporter gene. Therefore, 293T cells were plated in 6-well plates at 4×10^5 cells per well and transiently transfected by the calcium phosphate-DNA coprecipitation method. Each transfection contained 800 ng of the expression plasmids, as well as 100 ng of pNFconluc plasmid as reporter and 100 ng pUT651 plasmid as a reference for transfection efficiency. 24 hours after transfection, these cells were trypsinized and seeded on a 24-well plate. Another 24 hours later, cells were either stimulated with 1000 IU/ml hTNF, 20 ng/ml mIL1- β , 200 ng/ml TPA (Sigma) or left untreated. After 6 hours of stimulation, cells were lysed in 200 μ l lysis buffer and analyzed for luciferase and β -galactosidase activity as described in De Valck et al., 1997. GFP and GFP-A20 served as negative and positive controls, respectively.

[0097] Similar to A20, both splice variants of ABIN were able to block TNF or IL-1 induced NF- κ B activation in these cells, with the shorter N-terminal truncated isoform being slightly more effective (FIG. 4). Moreover, structure-function analysis of ABIN deletion mutants revealed that the NF- κ B inhibiting activity resides in the C-terminal 228 amino acids (ABIN(420-647) SEQ ID NO:10) which are also sufficient for interaction with A20. The latter ABIN-mutant no longer contains the leucine zipper structure, demonstrating that this protein domain is not involved in the interaction with A20 nor in the inhibition of NF- κ B (FIG. 4). Overexpression of a combination of suboptimal doses of A20 and ABIN, that on their own were not sufficient to inhibit NF- κ B activation, diminished NF- κ B activation upon stimulation with TNF (FIG. 5) or IL-1 considerably. This suggests that ABIN mediates the NF- κ B inhibiting effect of A20.

[0098] Total ABIN (1-647; SEQ ID NO:2), the shorter splice variant (54-647; SEQ ID NO:19) and the C-terminal fragment (390-647) are also able to block the TPA induced NF- κ B activation (FIG. 6).

[0099] Similar results were obtained when ABIN2 was used in the test instead of ABIN or ABIN-fragments (FIG. 7).

Example 6: Effect of ABIN and/or ABIN fragments on NF- κ B activation induced by overexpression of TRADD, RIP, TRAF2, NIK or p65.

[00100] Expression vectors for ABIN and ABIN fragments were constructed as described above. The expression vectors containing TRAF2, NIK and p65 have previously been described (Malinin et al., 1997; Rothe et al., 1994; Vanden Berghe et al., 1998). PCR fragments encoding TRADD and RIP were cloned in pCDNA3 (Invitrogen, Carlsbad, CA) in frame with a C-terminal E-tag. Transfection and reporter assay was carried out as described above.

[00101] NF- κ B can be activated in 293 T cells by TNF treatment as well as by overexpression of specific proteins of the TNF-receptor complex, including TRADD, RIP, TRAF2 and NIK (Rothe et al., 1995; Malinin et al., 1997; Hsu et al., 1995; Ting et al., 1996). The latter associates with and activates I κ B kinase complex which leads to I κ B phosphorylation. This is a signal for ubiquitination and degradation of I κ B, thus releasing NF- κ B which then translocates to the nucleus. Co-transfection of expression plasmids encoding these TNF-receptor associated proteins together with the expression plasmids encoding full length ABIN, showed that the latter completely inhibited NF- κ B activation induced by TRADD or RIP, and partially inhibited TRAF2-induced NF- κ B activation. In contrast, no clear difference was observed when NF- κ B-dependent reporter gene expression was induced by NIK or more directly by overexpression of the p65 subunit of NF- κ B (FIG. 8, FIG. 9). These results suggest that ABIN inhibits TNF-induced NF- κ B activation at a level preceding the activation of the NIK-I κ B kinase steps, for example at the level of TRAF2 in the TNF-receptor complex.

[00102] As members of the TRAF family mediate NF- κ B activation by several other stimuli, including IL-1, lymphotoxin β , CD30 and CD40 (Rothe et al., 1995; Cao et al., 1996; Nakano et al., 1996; Aizawa et al., 1997; Ishada et al., 1996), ABIN might have the potential to inhibit NF- κ B activation in response to a wide range of inducers. Therefore, drugs that mimic the activity of ABIN

are likely to have therapeutic value in inflammatory and neurodegenerative diseases as well as in cancer and AIDS.

Example 7: Cell transfection, Co-immunoprecipitation and Western blotting.

[00103] 2×10^6 human embryonic kidney 293T cells were plated on 10 cm Petri dishes and transiently transfected by calcium phosphate-DNA coprecipitation. 24 hours after transfection, cells were lysed in 500 l of lysis buffer (50 mM Hepes pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40 and 5 mM EDTA). Lysates were incubated with 5 l of rabbit anti-GFP antibody (Clontech) and immunocomplexes were immobilized on protein A-Trisacryl (Pierce). The latter were washed twice with lysis buffer and twice with lysis buffer containing 1M NaCl. Coprecipitating proteins were separated by SDS-PAGE and analyzed by Western blotting with mouse anti-E-tag antibody (Pharmacia).

Example 8: NF- κ B dependent reporter gene assay.

[00104] NF- κ B activity was determined by the NF- κ B dependent expression of a luciferase reporter gene. Therefore, 293T cells were plated in 6-well plates at 4×10^5 cells per well and transiently transfected by the calcium phosphate-DNA coprecipitation method. Each transfection contained 800 ng of the specific expression plasmids, as well as 100 ng of pNFconluc plasmid and 100 ng pUT651 plasmid. 24 hours after transfection, these transfectants were trypsinized and seeded on a 24-well plate. Another 24 hours later, cells were either stimulated with 1000 IU/ml hTNF or 7000 IU/ml IL-1 or left untreated. After 6 hours of stimulation, cells were lysed in 200 μ l lysis buffer and analyzed for luciferase and β -galactosidase activity. Luciferase values (luc) are normalized with (β -galactosidase values (gal) and plotted as luc/gal.

Example 9: Site Specific Mutagenesis.

[00105] Site specific mutagenesis on ABIN was performed by overlap PCR reaction using primers which contain the desired mutations. The primers used were the mutation primers 5'-GAATACCAAGGAGGCGCAGATCCAGCGGCTAATAAGCTTGGAGGAGGC-3' (SEQ ID NO:11), 5'-GTTGCTGAAAGAGGACGTAAAATCTTGAAGAGG-3' (SEQ ID NO:12), 5'-GCAGGTAAAAATCTTGAAGAGAATGCCAGAGGGAACG-3' (SEQ ID NO:13), and

5'-GCAGGTAAAAATCTTGAAGAGGACTTCCAGAGGAAAC

GGAGTGATGCGCAACGCATGCCG-3' (SEQ ID NO:14), a forward primer located at the start codon and two reverse primers, one hybridizing in the 3' UTR and one in the coding region. The Xhol-BstEII fragment of wild type ABIN(54-647) in pCAGGS was exchanged with the same fragment of the PCR amplified mutated ABIN cDNA's.

Example 10: Binding of ABIN with A20 is not sufficient for its NF-κB inhibiting potential.

[00106] A two hybrid assay with A20 revealed another novel A20-binding protein which was also able to inhibit NF-κB activation upon overexpression. BLAST searches with this novel protein, named ABIN-2, revealed no homology with any known protein. However, by comparison of the protein sequence of ABIN-2 with ABIN, two boxes of 19 (AA 423-441) and 21 (AA 475-495) amino acids long with 68% and 67% homology were identified respectively. Therefore, the contribution of these regions to the binding with A20 and to the NF-κB inhibiting effects of ABIN were analyzed by site specific mutagenesis of a number of conserved amino acids. Co-immunoprecipitation analysis after transient overexpression of GFP or GFP/A20 together with wild type ABIN or its site specific mutants (ABIN-MUT1, ABIN-MUT2, ABIN-MUT3 and ABIN-MUT4) (SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18 respectively) in 293T cells, showed that all of these mutants can still bind A20 (FIG. 10). On the other hand, point mutations in the second box (ABIN-MUT2, ABIN-MUT3 and ABIN-MUT4) completely abolished the ability of ABIN to block NF-κB activation upon stimulation of 293T cells with TNF, even when higher amounts of these expression plasmids were transfected. In contrast, the mutation in the first box (ABIN-MUT1) only slightly diminished the NF-κB inhibiting effect of ABIN (FIG. 11). Furthermore, point mutations in the second conserved motif dominantly interfered with the NF-κB inhibiting effect of wild type ABIN (FIG. 12). ABIN-MUT2 and ABIN-MUT3 exhibit a more potent function as dominant negative mutants of ABIN, compared to ABIN-MUT4. In these assays, comparable expression levels of the different mutants and of wild type ABIN were obtained as judged by Western blot analysis using anti-E tag antibody. These results suggest that the second conserved region is involved in the NF-κB inhibiting effects of ABIN, and that binding of ABIN with A20 as such is not sufficient for inhibition of NF-κB activation.

REFERENCES

- Aizawa S, et al. Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NF- κ B activation. *J Biol Chem.* 272, 2042-2045. (1997)
- Barnes PJ, et al. Anti-inflammatory actions of steroids : molecular mechanisms. *Trends Pharmacol Sci.* 14(12) : 436-441 (1993).
- Barnes PJ and Karin M. Nuclear factor-kappaB : a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.* 336(15) : 1066-1071 (1997).
- Behl C, et al. Mechanism of amyloid beta protein induced neuronal cell death : current concepts and future perspectives. *J Neural Transm Suppl.* 49 : 125-134 (1997).
- Brand K, et al. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest.* 97(7) : 1715-1722 (1996).
- Brand K, et al. Dysregulation of monocytic nuclear factor-kappa B by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol.* 17(10) : 1901-1909 (1997).
- Cao Z, et al. TRAF6 is a signal transducer for interleukin-1. *Nature.* 383, 443-446. (1996)
- De Valck D, et al. A20, an inhibitor of cell death, self-associates by its zinc finger domain. *FEBS Lett.* 384, 61-64. (1996)
- De Valck D, et al. A20 inhibits NF- κ B activation independently of binding to 14-3-3 proteins. *Biochem Biophys Res Commun.* 238, 590-594. (1997)
- DiDonato JA, et al. A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature.* 388, 548-554. (1997)

Dixit VM, et al. Tumor necrosis factor-alpha induction of novel gene products in human endothelial cells including a macrophage-specific chemotoxin. *J Biol Chem.* 265, 2973-2978. (1990)

Harris M. From negative factor to a critical role in virus pathogenesis : the changing fortunes of Nef. *J Gen Virol.* 77, 2379-2392. (1996)

Hsu H, et al. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell.* 81, 495-504. (1995)

Ishida T, et al. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J Biol Chem.* 271, 28745-28748. (1996)

Jaattela M, et al. A20 zinc finger protein inhibits TNF and IL-1 signaling. *J Immunol.* 156, 1166-1173. (1996)

Kaltschmidt C, et al. Transcription factor NF-kappa B is activated in microglia during experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 55(1) : 99-106. (1994)

Kimura A, et al. Detailed analysis of the mouse H-2K^b promoter : enhancer-like sequences and their role in the regulation of class I gene expression. *Cell* 44(2) : 261-272. (1986)

Kopp EB and Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science.* 265(5174) : 956-959. (1994)

Krikos A, et al. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by κB elements. *J Biol Chem.* 267, 17971-17976. (1992)

Luque I, et al. Rel/NF-kappa B and I kappa B factors in oncogenesis. *Semin Cancer Biol.* 8(2) : 103-111. (1997)

Malanin NL, et al. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. Nature. 385, 540-544 (1997)

Mosialos G. The role of Rel/NF-kappa B proteins in viral oncogenesis and the regulation of viral transcription. Semin Cancer Biol. 8(2) : 121-129. (1997)

Nagase, T et al. Prediction of the coding sequences of unidentified human genes. III. The coding sequences of 40 new genes (KIAA0081-KIAA0120) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res.2(1), 37-43 (1995).

Nakano H, et al. TRAF5, an activator of NF-κB and putative signal transducer for the lymphotoxin-(receptor. J Biol Chem. 271, 14661-14664. (1996)

Niwa H. et al. Efficient selection for high-expression transfecants with a novel eukaryotic vector. Gene. 108, 193-199. (1991)

OPIPARI, A.W. et al.. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. J. Biol. Chem. 265, 14705-14708, (1990).

Pahl HL, et al. The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kappaB. J Exp Med. 183(4) : 1829-1840. (1996)

Regnier CH, et al. Identification and characterization of an IκB kinase. Cell. 90, 373-383. (1997)
Remick DG. Applied molecular biology of sepsis. J Crit Care. 10(4) : 198-212. (1995)

Rothe M, et al. TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. Science. 269, 1424-1427. (1995)

Rothe M, et al. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. 78, 681-692. (1994)

Schreck R, et al. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J*. 10(8) : 2247-2258 (1991)

Song HY, et al. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF- κ B activation. *Proc Natl Acad Sci USA*. 93, 6721-6725. (1996)

Tewari et al., Lymphoid expression and regulation of A20, an inhibitor of programmed cell death; *J.Immunol.* 1995, 154: 1699-1706.

Ting AT, et al. RIP mediates tumor necrosis factor receptor 1 activation of NF- κ B but not Fas/APO-1-initiated apoptosis. *EMBO J*. 15, 6189-6196. (1996)

Vanden Berghe W. Et al. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 273 (6) : 3285-3290. (1998)

Wang P, et al. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J Biol Chem*. 270(16) : 9558-9563. (1995)

Woronicz JD, et al. I κ B kinase-beta : NF- κ B activation and complex formation with I κ B kinase- α and NIK. *Science*. 278, 866-869.(1997)

Yeh WC. Et al. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* 7(5) : 715-725. (1997)

Zandi E, et al. The I κ B kinase complex (IKK) contains two kinase subunits, IKK α and IKK β , necessary for I κ B phosphorylation and NF- κ B activation. Cell. 91, 243-252. (1997)